Effects of Refrigeration and Freezing on the Electromechanical and Biomechanical Properties of Articular Cartilage

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In vitro electromechanical and biomechanical testing of articular cartilage provide critical information about the structure and function of this tissue. Difficulties obtaining fresh tissue and lengthy experimental testing procedures often necessitate a storage protocol, which may adversely affect the functional properties of cartilage. The effects of storage at either 4 °C for periods of 6 days and 12 days, or during a single freeze-thaw cycle at −20 °C were examined in young bovine cartilage. Non-destructive electromechanical measurements and unconfined compression testing on 3 mm diameter disks were used to assess cartilage properties, including the streaming potential integral (SPI), fibril modulus (Ef), matrix modulus (Em), and permeability (k). Cartilage disks were also examined histologically. Compared with controls, significant decreases in SPI (to 32.3 ± 5.5% of control values, p < 0.001), Ef (to 3.1 ± 41.3% of control values, p = 0.046), Em (to 6.4 ± 8.5% of control values, p < 0.0001), and an increase in k (to 26767 ± 2562.0% of control values, p = 0.004) were observed at day 12 of refrigeration at 4 °C, but no significant changes were detected at day 6. A trend toward decreasing a decrease in SPI (to 94.2 ± 6.2% of control values, p = 0.083) was identified following a single freeze-thaw cycle, but no detectable changes were observed for any biomechanical parameters. All numbers are mean ± 95% confidence interval. These results indicate that fresh cartilage can be stored in a humid chamber at 4 °C for a maximum of 6 days with no detrimental effects to cartilage electromechanical and biomechanical properties, while one freeze-thaw cycle produces minimal deterioration of biomechanical and electromechanical properties. A comparison to literature suggested that particular attention should be paid to the manner in which specimens are thawed after freezing, specifically by minimizing thawing time at higher temperatures.

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1 Introduction

The composition and highly specific organization of articular cartilage, composed of a hydrated proteoglycan matrix trapped in a primarily type II collagen network, allow this tissue to withstand and distribute applied mechanical loads. When cartilage is compressed, interstitial fluid attempts to flow out of the tissue through the dense proteoglycan matrix, thereby pressurizing the fluid and allowing for load-bearing [1–4]. The collagen fibers entrapping and retaining the proteoglycan matrix are placed under tension and restrain the tissue from expansion [5,6]. Biomechanical and electromechanical tests are used to assess cartilage functional properties and reflect its composition and structure [7–12]. The limited availability of fresh animal or human articular cartilage and the lengthy testing procedures employed in some experimental protocols often require that cartilage is stored before or during an experiment. Understanding the effects of storage, which often include refrigeration or freezing, on biomechanical and electromechanical properties is critical to correctly designing testing protocols and interpreting their results.

Optimizing articular cartilage storage at 4 °C has been of interest for preserving allograft material [13–15]. These studies have shown that chondrocyte viability can be maintained up to 14 days [15,16], while glycosaminoglycan (GAG) content and biomechanical properties, measured in indentation, confined compression, and tensile testing, remain unchanged up to 28 days [14]. Thomas et al. [17] performed sequential indentation testing at multiple time points on the same joint surfaces and reported no changes after 4 days of 4 °C storage. In these studies, osteochondral plugs were typically stored in a serum-free culture medium that was sometimes supplemented with antibiotics or other additives [13–15,17]. Charlebois et al. [18] described a method of creating humid chambers that permitted chondrocytes to remain viable for up to 2 weeks at 4 °C. In summary, articular cartilage has been shown to retain certain biomechanical properties and biochemical composition up to 28 days, and cell viability and density up to 14 days, during refrigerated storage.

Cartilage biomechanical properties, determined in indentation testing [19,20] and confined compression [21], have been shown to degrade following freezing. However, several studies [22–24] and anecdotal observations [25,26] suggest that no changes in indentation properties occur due to freezing. Confined compression tests of osteochondral plugs, before and after storage at −20 °C immersed in phosphate buffered saline (PBS), resulted in statistically significant decreases in aggregate modulus [21]. Kennedy et al. [20] reported that cartilage indentation stiffness decreased in whole bovine joints either frozen at −20 °C or flash-frozen to −80 °C. However, creep tests of individual condyles after freezing at 0 °C resulted in no change in instantaneous shear modulus but a statistically significant decrease in relaxed shear modulus and increase in creep rate, indicating that the ability to resist sustained loading was compromised [19,27]. Similarly, indentation testing performed on osteochondral cores at physiological stress levels showed no effect due to freezing at −80 °C with or without dimethyl sulfoxide as a protectant [23], and whole
human femoral heads tested prior to and following freezing at −20°C did not result in changes in the response to indentation testing [22]. In summary, several studies have reported substantial losses in biomechanical properties in articular cartilage subjected to a single freeze-thaw cycle, while others report the contrary; that the biomechanical properties of cartilage are not altered as a result of freezing.

Due to its composition and structure, articular cartilage exhibits electromechanical properties [9,28–31]. Fixed negative charges on the sulfate and carboxylate groups of the proteoglycans entrapped in the collagen network are balanced by mobile positive ions in the interstitial fluid. When cartilage is compressed fluid flows out of the tissue displacing the mobile positive ions relative to the fixed negative charges generating streaming potentials. Streaming potentials reflect cartilage composition and function and are sensitive to degradative changes [7–10,32,33]. In general, degraded cartilage, which is typically proteoglycan depleted [34], yields lower streaming potentials compared with proteoglycan-rich normal articular cartilage [9,10,30,32,33]. To date, the effect of refrigerated or frozen storage on the electromechanical properties of articular cartilage has not been reported in the literature.

The current data summarized above indicate variable results with respect to the effect of freezing or refrigeration on the biomechanical properties of articular cartilage, and that the effects of storage on electromechanical properties are unknown. In the current study the effects of these commonly used storage temperatures on biomechanical and electromechanical properties of articular cartilage were examined. We included refrigeration at 4°C in humid chambers for 6 days or 12 days, and freezing at −20°C followed by thawing just prior to testing (one freeze-thaw cycle). We hypothesized that, with respect to both electromechanical and biomechanical properties, (1) storage at 4°C for 6 days would cause detectable changes, (2) storage at 4°C for a period of 12 days would lead to more substantial changes than those observed by 6 days, and (3) a single freeze-thaw cycle would result in diminished properties.

2 Methods

2.1 Sample Preparation and Experimental Design. A young bovine stifle (knee) joint, approximately 6 months of age, was received fresh from a local butcher. The medial and lateral condyles were stored at 4°C in humid chambers overnight. Humid chambers were created by placing the joint surface into an airtight container with saline-soaked kimwipes arranged so that they were not in direct contact with the articular surface [18,35].

Four regions on each condyle were identified using small diameter pins, placed such that there was sufficient joint surface around each pin to allow for non-destructive electromechanical measurements at eight sites and subsequent removal of four 3.5 mm diameter osteochondral cores at a subset of these sites for biomechanical testing and histology. On each condyle, two sites around each pin, labeled “control” (n=8), were tested on day 1 prior to storage and two were labeled “storage,” corresponding either to storage at 4°C or −20°C (n=8). The remaining four sites around each pin were used for sequential electromechanical testing, where electromechanical measurements could be made at all time points throughout the experiment (Fig. 1). In the refrigeration experiment, the medial femoral condyle was placed in a humid chamber at 4°C during the intervals between days 1 and 6 and days 6 and 12. In the freezing experiment, the lateral femoral condyle was frozen at −20°C for 7 days immediately following day 1 measurements. On day 7, it was thawed at room temperature in PBS solution (Sigma-Aldrich, St. Louis, MO) for approximately 30 min prior to testing.

2.2 Electromechanical Measurements. The Arthro-BST™, an arthroscopic device for cartilage assessment (Biomomentum Inc., Laval, QC, Canada), was used to non-destructively measure the electromechanical properties of cartilage during indentation. This hand-held tool records the streaming potential distribution generated during a light cartilage indentation, and calculates a quantitative parameter, the streaming potential integral (SPI) (mV*mm^3), at a standardized amplitude of compression of 150 µm. Electrical signals were measured by an array of 37 gold microelectrodes equally distributed on the hemispherical tip (radius of curvature=3.05 mm) of the Arthro-BST™ device. A digital camera and software assisted users in identifying measurement positions and minimizing error due to positioning the Arthro-BST™ device at each predefined location on the articular surface. Two users measured each site three times for a total of six electromechanical measurements per site. The joint surface was immersed in PBS solution for approximately 30 min prior to beginning measurements with the Arthro-BST™.

2.3 Unconfined Compression Testing. Osteochondral cores, isolated from the joint surface using a 3.5 mm diameter mosaic-plasty punch (Smith & Nephew, Andover, MA), were stored in individual humid chambers at 4°C for up to 24 h. Each sample was tested in unconfined compression geometry using a Mach-1™ micromechanical tester (Biomomentum Inc., Laval, QC, Canada). Immediately prior to testing, the cartilage was separated from the underlying bone using a scalpel, repunched with a 3 mm diameter biopsy punch, and immersed in PBS for 15 min. Cartilage disk thickness was measured with an upright digital calliper (Mitutoyo, Kawasaki, Japan) and used to calculate the testing parameters, which consisted of five compression ramps of 2% strain applied at a rate of 0.4% strain/s. Between ramps, the cartilage was allowed to relax until the load decay was 0.01 g/min. The fibril-network reinforced biphasic model was fitted to the data to obtain fibril modulus (Ef), matrix modulus (Em), and permeability (k) [6,36,37].

2.4 Histology. After biomechanical testing, cartilage disks were fixed in 5% glutaraldehyde, 0.1M sodium cacodylate, and embedded in paraffin. 5 µm sections were stained with Safranin-O/Fast Green.

2.5 Statistical Analysis. SPI measurements were analyzed separately for each experiment with respect to user, day, and measurement repetition using a general linear model repeated measures analysis of variance (ANOVA) that permitted interactions between user and day, and user and measurement repetition. In the
3 Results

3.1 Electromechanical Measurements. Electromechanical changes were detected in the refrigeration experiment, where a statistically significant decrease was found between days 1 and 12 ($p < 0.001$, Fig. 2(a)), representing an average decrease to $32.3 \pm 5.5\%$ (mean $\pm$ 95% confidence interval (CI)) of day 1 SPI values. No significant change occurred following 6 days of refrigerated storage, and SPI values were $101.2 \pm 7.5\%$ (mean $\pm$ 95% CI) of day 1 values (Fig. 2(a)). In the freezing experiment, a trend toward a significant change was detected indicating a tendency for SPI to decrease following a single freeze-thaw cycle to an average of $94.2 \pm 6.2\%$ (mean $\pm$ 95% CI) of day 1 values, ($p = 0.083$, Fig. 2(b)). We do not consider this decrease to be biologically significant for in vitro experiments of cartilage electromechanical and biomechanical properties.

An effect of measurement repetition ($p = 0.001$) was detected in the refrigeration group. A decrease in SPI with measurement repetition was detected at all time points, but was more pronounced at day 12, reflecting the reduced ability of degraded tissue to recover from indentation in the short time (on the order of several minutes) permitted between measurements. This can be appreciated as an increase in the average coefficient of variation from a maximum of 15% for day 1 and 6 SPI measurements compared with approximately 30% by day 12. The effect of measurement repetition was secondary and inconsequential compared with the very significant overall effect of storage time.

3.2 Biomechanical Parameters. $E_f$, $E_m$, and $k$, determined from the model fits of the fifth stress relaxation ramp for each sample, were compared. By day 12, $E_f$ decreased to $3.1 \pm 41.3\%$ (mean $\pm$ 95% CI) and $E_m$ to $6.4 \pm 8.5\%$ (mean $\pm$ 95% CI) of day 1 values in a statistically significant manner with $p = 0.046$ and $p < 0.0001$ for $E_f$ and $E_m$, respectively (Fig. 3). There were no significant differences detected between days 1 and 6 in the refrigeration group or following freezing for these two parameters. Permeability ($k$) increased to $2676.7 \pm 2562.0\%$ (mean $\pm$ 95% CI) by day 12 of refrigerated storage compared with controls ($p = 0.004$). However, there was no change in permeability after 6 days of refrigerated storage or as a result of freezing. Cartilage thickness was constant in all sample groups, ranging from approximately 1.5 mm to 2.5 mm.

4 Discussion

Understanding the effects of frozen or refrigerated storage on articular cartilage is essential for proper protocol design and the interpretation of experimental findings where storage is often required. Our first hypothesis, that storing cartilage in humid chambers at $4^\circ C$ for 6 days would result in detectable changes, was disproved. Electromechanical and biomechanical properties were maintained (Figs. 2(a) and 3) and these findings were supported by Safranin-O/Fast Green staining, where day 6 samples were similar to controls (Fig. 4). Our second hypothesis was supported by our experimental results, as statistically significant changes in SPI and biomechanical properties were observed after 12 days of refrigerated storage, indicative of weakened cartilage tissue that was substantially less stiff than day 6 or day 1 controls (Figs. 2(a) and 3). Safranin-O/Fast Green staining revealed a near-complete loss of proteoglycan in these samples (Figs. 4(c) and 4(f)). Our third hypothesis, that a single freeze-thaw cycle would result in severely diminished properties, was not supported. While a trend toward a statistically significant decrease in SPI was observed, these changes were small ($5.8 \pm 6.2\%$ decrease compared with day 1), and no changes in biomechanical properties were detected (Figs. 2(b) and 3). No pattern of histological changes was detected in Safranin-O/Fast Green stained sections of frozen specimens.

4.1 Refrigeration up to 6 days in Humid Chambers Maintains Biomechanical and Electromechanical Properties of Articular Cartilage. In the refrigeration group, significant changes in electromechanical (Fig. 2(a)), biomechanical (Fig. 3), and histological (Fig. 4) properties were observed at day 12 but no changes were detected at day 6. This preservation of properties for a duration of 6 days is less than other reports [13–16], and this difference can be attributed to several factors. The most influential
factor is likely the various methods in which the samples were stored while at 4°C. We selected a relatively simple storage method, the humid chamber in contrast to the use of tissue culture media and conditions explored by investigators looking to preserve allograft tissue. Maintaining cartilage in tissue culture conditions improved chondrocyte viability, and appeared to allow recovery in Safranin-O staining by 28 days to baseline levels after a depleted superficial zone was observed at 14 days. In addition, disparities between our findings and those reported in the literature may result from the baseline to which comparison is made. For example, two studies reported that cartilage compressive, tensile, and indentation properties were maintained up to 28 days; however, these conclusions were made by comparing samples stored for 7 versus 28 days, since pre-storage biomechanical testing was not performed.

Our findings indicate that gradual tissue degradation is occurring between days 6 and 12 of refrigeration in humid chambers, which may be due to gradual cell death resulting in the release of degradative enzymes. This is earlier than the reported maximum of 2 weeks that cell viability was maintained in cartilage refrigerated in human chambers, but comparable to the maintenance of metabolic activity for 5 days in osteochondral samples stored at 4°C submerged in a nutrient solution. The underlying bone present in our samples may have provided a source of degradative factors as it was not irrigated nor immersed in culture media. The substantial decrease in SPI observed at all sites on the joint surface at day 12 reflects a loss of proteoglycan, a relationship that is well documented. Degradation is confirmed by changes in the parameters of the fibril-network-reinforced biphasic model, which are used to fit the stress relaxation ramps from unconfined compression testing, and which reflect the two major extracellular matrix constituents of cartilage. Specifically, Em is related to the stiffness of the drained proteoglycan matrix, and Ef to the stiffness of the collagen fiber network. The significant decreases in these two parameters indicate a weakening of the collagen network and loss of proteoglycan molecules, both of which are essential for load-bearing.

4.2 One Freeze-Thaw Cycle Exerts a Nominal Influence on Electromechanical But No Detectable Changes in Biomechanical Properties of Articular Cartilage. A single freeze-thaw cycle caused a trend toward a significant but small decrease in cartilage electromechanical properties, but did not cause changes in biomechanical properties nor any pattern of histological

![Fig. 3](image-url)
changes. Taken together, it appears that one freeze-thaw cycle may lead to minor degradation to the extracellular matrix, which can be detected by sensitive electromechanical measurements, but not by biomechanical or histological assessment methods.

Our biomechanical findings related to the effect of freezing agree with those of several studies [22–24,26] but are contrary to the considerable reductions in material properties for indentation [19,20] and confined compression tests [21] reported by other investigators. A possible reason for these discrepancies in the literature may be due to differences in how investigators have treated their samples with respect to thawing and the time between thawing and mechanical testing. For example, Kennedy et al. [20] froze whole joints with intact soft tissues and allowed them to thaw overnight at room temperature prior to testing. Willett et al. [21] froze osteochondral dowels immersed in PBS at −20°C and thawed them in a 37°C water bath, and Black et al. [19] froze samples to 0°C for 5 min before thawing and incubating in media for 10 days at 37°C. We speculate that it is possible that in these studies, where a substantial reduction in mechanical properties was detected, the considerable time spent at elevated temperatures provided a greater opportunity for degradative enzymes to operate [46,47]. In contrast, Kiefer et al. [23], who reported that freezing caused no change in cartilage indentation properties, employed a shorter thawing procedure in which samples were submerged in PBS for 15 min and then incubated in Ham’s solution for 2 h at 37°C. In our study, the frozen joint surface was submerged in room temperature PBS for approximately 30 min before beginning electromechanical measurements. This is a comparatively short thawing procedure, which may have limited time for enzymatic degradation to occur, although this was not directly assessed. Based on these observations, we recommend that frozen cartilage be thawed rapidly and subsequent mechanical tests conducted with minimal delay in order to limit degradative changes in the extracellular matrix.

4.3 Conclusions. Our findings indicate that joint surfaces can be stored in humid chambers at 4°C up to 6 days without experiencing significant changes in cartilage electromechanical and biomechanical properties while storage for longer times can induce significant and biologically important degradation. A single freeze-thaw cycle produced small but detectable changes in electromechanical properties but not in biomechanical properties. Streaming potentials provide a sensitive, nondestructive method for rapid cartilage assessment that is user independent. Future studies comparing different thawing protocols and the treatment of samples between thawing and mechanical testing could perhaps explain the discrepancies observed in the literature concerning the effect of freezing on cartilage biomechanical properties.

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